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Experimental *Yersinia enterocolitica* infection in mice: Kinetics of growth

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Infection of several strains of laboratory mice with a virulent strain of *Yersinia enterocolitica* was followed by performing viable bacterial counts on homogenates of selected tissues at intervals after intragastric, aerogenic, or intravenous infection. It is observed that CD-1 mice are more susceptible to *Y. enterocolitica* infection than either the C_{57}B1/6 or B6D2 strains. Development of an enteric infection is dose dependent; less than 5×10^7 organisms by mouth yields sporadic, low levels of systemic infection, with many of the animals showing no apparent infection. Increasing the challenge inoculum by a factor of 10 eliminates the variability among the animals, giving rise to an enteric infection in all of the mice that moves quickly to the mesenteric lymph node. The bacterial population in the lymph node multiplies rapidly, and the infection is disseminated to the spleen, liver, and lungs, ultimately killing most of the animals. Exposure to an aerogenic challenge of less than 1,000 organisms resulted in a fulminating pneumonitis with an invariably fatal outcome. Intravenous challenge with 500 organisms caused a rapidly fatal, systemic infection. The growth of the bacteria in the intravenously infected mouse depends upon the temperature at which the challenge inoculum had been grown in vitro. At temperatures below 26 C, the bacteria are cleared from the blood at a slower rate and are more resistant to intracellular killing, as compared to organisms grown at 37 C. This effect results in the inoculum increasing to greater numbers in the tissues in a shorter period of time.

Yersinia enterocolitica is being increasingly recognized as a probable cause of acute gastroenteritis in man (13, 15, 18). Furthermore, such chronic, systemic diseases as Reiter syndrome, erythema nodosum, arthritis, oseitis, and others (15) may be sequelae to a primary *Yersinia* infection that may have been either severely debilitating or subclinical in nature. There is a large body of circumstantial evidence that *Y. enterocolitica* is pathogenic for man (12), but it has not been possible to confirm early reports (19) of laboratory animal infection until quite recently (4; Quan et al., J. Infect. Dis., in press).

A current study from this laboratory (P. B. Carter, submitted for publication) showed striking similarities between the pathology observed in laboratory animals infected with *Y. enterocolitica* strain WA and that seen in some human cases. Therefore, the present work was undertaken to gain further basic information on the kinetics of bacterial growth in the normal mouse after challenge by various routes. Such data confirm that a valid laboratory animal model of the human disease does in fact exist, and the immune responses to this infection may now be studied experimentally. This model should also prove useful in testing the efficacy of various *Y. enterocolitica* vaccines and therapeutic regimens.

MATERIALS AND METHODS

Animals. Six- to eight-week-old male and female random-bred, specific pathogen-free, CD-1 (Charles River, Wilmington, Mass.), inbred C_{57}B1/6 (Jackson Laboratories, Bar Harbor, Me.) and B6D2 (C_{57}B1/6 $\times$ DBA/2 F_1) mouse strains were used. The animals were maintained 10 to a cage with free access to food (Charles River Rat/Mouse Formula, Country Foods, Syracuse, N.Y.) and water.

Bacteria. The two strains of *Y. enterocolitica* used have been described in detail elsewhere (4). The WA strain (ATCC no. 27729 and NCTC no. 10938) was recently isolated from the blood of a human patient and found to be highly pathogenic for mice (4). Strain 5819, which is avirulent for mice, was kindly supplied by the New York State Department of Health, Albany.

Bacterial enumeration technique. Groups of five randomly selected mice were ether-anesthetized and

exsanguinated from the exposed heart, and the blood immediately was surface-plated on Trypticase soy agar (TSA) (BBL, Cockeysville, Md.). The lung, liver, spleen, and mesenteric lymph nodes were then removed aseptically and homogenized separately in saline as previously described (6), and appropriate saline dilutions were surface-plated on TSA. After 36 h of incubation at 37 C, the typical small translucent colonies of *Y. enterocolitica* were counted. Doubtful colonies were tested by slide agglutination using hyperimmune mouse serum. The minimal detectable limits of this technique are 20 colony-forming units per ml of blood, 50 per spleen, and 100 for liver, lung, and mesenteric lymph node.

Animal challenge. The methods of animal challenge have been described by Collins and Carter (9). Mice receiving an aerogenic challenge were exposed to aerosols of a young *Y. enterocolitica* broth culture (diluted in saline to contain 10^7 viable organisms/ml) for 30 min in a Middlebrook chamber (Tri-R instruments, Rockville Center, N.Y.). Intragastric inocula were introduced directly into the stomach with a 19-gauge, stainless-steel feeding needle (Popper and Sons, New York, N.Y.). Intravenously challenged mice received a suitable saline dilution of a young *Y. enterocolitica* broth culture in 0.1 ml of saline via the left lateral tail vein. In all cases, the number of viable bacilli in the challenge inocula was determined by plating saline dilutions on TSA immediately after the infection procedures.

Peritoneal clearance. Peritoneal clearance and killing was observed in CD-1 mice injected intraperitoneally with 0.2 ml of a saline suspension of viable *Y. enterocolitica* WA cells that had been opsonized 30 min earlier with a 1:10 saline dilution of hyperimmune mouse serum. Three minutes later, the peritoneal cavity was washed out with 2.5 ml of Hanks balanced salt solution containing 1% fetal calf serum (Gibco, Grand Island, N.Y.). The washout from each animal was then diluted 1:20 in cold Hanks balanced salt solution in plastic tubes. One tube was immediately taken for the time zero count, and the others were placed in a 37 C water bath shaker. At intervals, tubes were removed and chilled immediately in an ice-water bath, and the ratio of cell-associated to cell-free yersiniae was determined (1).

RESULTS

Oral Y. enterocolitica WA infection. None of the mice was susceptible to intragastric infection with strain 5819, but is was possible to infect CD-1, C_{57}B1/6, and B6D2 mice orally with 3×10^7 *Y. enterocolitica* WA, a dose equivalent to 0.1 mean lethal dose values (4). Severe systemic infections and death occurred in some of the animals, but many others demonstrated only minimal infections when tested at this dose level. The sporadic nature of the oral infection was overcome by increasing the challenge dose to 5×10^8 viable bacteria (Table 1). The *Yersinia* quickly infected the mesenteric lymph node, and the bacterial population progressively increased in numbers at that site.

TABLE 1. *Oral infection of CD-1 with 5×10^8 Y. enterocolitica WA*

Time postinfection (days)	Animal no.	Deaths	Log no. of bacteria/organ			
			Spleen	Liver	Mesenteric lymph node	Lung
1	1		—[a]	—	3.1	—
	2		—	—	2.3	—
	3		—	—	—	—
	4		—	—	2.3	—
	5		—	—	—	—
2	6		—	—	5.9	—
	7		—	—	5.8	—
	8		—	—	5.7	—
	9		—	—	4.7	—
	10		4.0	4.7	3.9	—
4	11	1	—	—	7.0	—
	12		6.2	—	6.8	—
	13		—	—	6.4	—
	14		—	—	6.1	—
	15		—	—	4.6	3.4
5		1				
6	16		6.4	—	8.2	6.8
	17		—	—	8.0	2.3
	18		—	—	7.5	4.6
	19		6.3	5.4	7.0	6.7
	20		—	—	7.0	2.0
7		2				
8	21	4	5.2	—	7.8	—
	22		—	—	6.8	—
	23		—	—	6.8	—
	24		—	—	6.5	—
	25		—	—	—	5.1
11	26	1	8.2	7.9	8.4	8.0
	27		5.1	6.8	8.3	7.2
	28		4.5	3.0	8.0	4.3
	29		3.6	3.2	7.9	3.3
	30		8.4	4.5	6.2	7.2

[a] No growth detectable.

From the infectious foci in the mesenteric node, organisms spread to the spleen, liver, and lungs to varying degrees in different animals. Death occurred from day 4 onward, with peak mortality on day 8. The fact that yersiniae did not appear in the lung until after day 4 indicated that the lung was not directly infected during the intragastric challenge procedure (9).

Because a number of animals succumbed to the infection between days 6 and 8, animals necropsied on days 8 and 11 may represent a group in which visceral involvement was somehow slowed or delayed; hence the lower lung

and mesenteric node counts seen on day 8 do not necessarily represent an immune response.

Aerogenic infection with Y. enterocolitica WA. Although human infection is primarily of gastrointestinal origin, the possibility of airborne infection (Pohl, as cited in reference 15) prompted an investigation of the aerogenic route of challenge in normal mice. Both CD-1 and B6D2 mice were simultaneously exposed to the same aerogenic challenge inoculum. However, the CD-1 mice were more uniformly infected and exhibited higher bacterial counts than the B6D2 mice starting as early as 1 h postchallenge. A progressive pulmonary infection developed over the next few days (Table 2), which rapidly disseminated to the liver and spleen between days 3 and 6. The extent of the infection in the liver and spleen did not always relate directly to the number of viable bacteria in the lungs because some mice carried large numbers of yersiniae in their lungs without any apparent involvement of the liver and spleen. Late in the infection only two mice (animals numbered 24 and 25, Table 2) failed to show detectable numbers of bacteria in the lung; the significant *Yersinia* counts in the spleens, however, indicated that the animals had been successfully infected by the challenge inoculum.

Almost all of the CD-1 mice that eventually succumbed to the aerogenic infection with *Y. enterocolitica* WA (Table 2) died between days 6 and 9; only six mice were available for the day 9 count. Hence, animals numbered 21 to 25 in Table 2 may again be considered a select group, and their lung counts may not represent the usual progress of the aerogenic infection. It is not clear whether the decreased bacterial counts in the livers and lungs of these mice are due to elimination of the infectious agent by the host defenses or are the result of a more slowly developing or aberrant infection in some of the mice.

Fewer deaths were observed among the aerogenically challenged B6D2 mice, even though peak bacterial populations in their tissues were comparable with those observed in the CD-1 mice. This may indicate a greater resistance to the lethal effects of *Y. enterocolitica* infection by the B6D2 strain of mouse.

Intravenous Y. enterocolitica WA infection. None of the mice had been susceptible to intragastric infection with *Y. enterocolitica* 5819 so the infection study was repeated using the intravenous route of inoculation. No growth of the organism was observed over the first 6 h in CD-1 mice infected intravenously with 6×10^5 *Y. enterocolitica* 5819 (Fig. 1). The viable

TABLE 2. *Aerogenic infection of CD-1 mice with Y. enterocolitica WA*

Time post infection	Animal no.	Deaths	Log no. of bacteria/organ		
			Spleen	Liver	Lung
1 h	1				2.9
	2				2.8
	3				2.7
	4				2.5
	5				2.5
1 day	6		—[a]	—	4.8
	7		—	—	4.8
	8		—	—	4.7
	9		—	—	4.7
	10		—	—	4.0
3 days	11		—	—	7.3
	12		—	—	7.2
	13		—	—	6.8
	14		—	—	6.6
	15		—	—	6.4
6 days	16	6	5.6	5.1	8.6
	17		—	3.6	8.4
	18		—	5.9	7.9
	19		—	2.3	7.8
	20		—	2.3	7.6
7 days		2			
8 days		2			
9 days	21	4	—	—	7.7
	22		—	—	7.4
	23		—	—	7.1
	24		3.9	—	—
	25		3.0	—	—
13 days		1			

[a] No growth detectable.

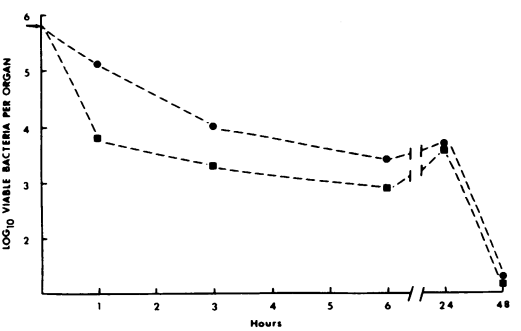

FIG. 1. *Growth of an intravenous challenge dose of Y. enterocolitica 5819 in CD-1 mice. Symbols: ●, liver populations; ■, the spleen populations. The arrow indicates the size of the challenge inoculum.*

counts for the liver and spleen decreased steadily, and the organisms were undetectable in vivo by 48 h. It was, however, possible to infect CD-1, $C_{57}B1$, or B6D2 mice by this route using as few as 100 viable *Y. enterocolitica* WA. The lowest challenge doses gave rise to sporadic systemic infections with some mice developing large populations of *Yersinia* in their tissues, whereas others had very few. As was seen in the oral and aerogenic infections, the $C_{57}B1$ and B6D2 mice contained fewer bacteria in their tissues than the CD-1 mice after the intravenous infection. The intravenous challenge of CD-1 mice with small doses of virulent yersiniae infected all of the mice; however, most of the organisms localized in the spleen. As the infection progressed, the liver and lungs then became more heavily infected. The large numbers of bacteria found in these tissues by day 8 were mostly localized within a few large abscesses rather than in the multiple small foci of infection usually seen in salmonellosis (14).

When the challenge inoculum was increased to 10^3 viable *Y. enterocolitica* WA, the resulting infection was very uniform so far as the percentage of animals infected and the numbers of bacteria found in the various tissues examined. The number of viable bacteria in the liver and spleen increased quickly and animals began to die 6 days postinfection (Fig. 2). Infection in the lungs lagged some 2 days behind the liver and spleen but was easily detectable by day 3.

Effect of culture temperature on virulence. In the preceding experiments, the challenge inocula were grown at 37 C. Because several differences between *Y. enterocolitica* WA cultured at 25 and at 37 C had been noted in previous work (4), the influence of temperature on mouse virulence was also studied. A fresh culture of *Y. enterocolitica* WA grown at 25 C was subcultured into two tubes of broth; one was incubated overnight at 37 C and the other at 25 C. When CD-1 mice were challenged intravenously with the respective cultures, a difference in the in vivo growth behavior over the first 2 h was apparent. The organisms grown at 37 C were cleared from the blood of normal mice very quickly, accumulating primarily in the liver and spleen (Fig. 3). The viable counts then fell precipitously over the first 2 h. Despite this fact, the surviving bacilli eventually grew rapidly in vivo and reached high counts in the test organs. On the other hand, organisms cultured at 25 C were cleared more slowly from the blood, which was still heavily infected after 2 h (Fig. 4). The bacterial populations in the liver, lung, and spleen did not decline appreciably after the first few minutes (Fig. 4), and over the following days the viable counts increased sharply (Fig. 5). The growth rates of both the 37 and 25 C grown *Y. enterocolitica* populations appeared to be about the same once the sys-

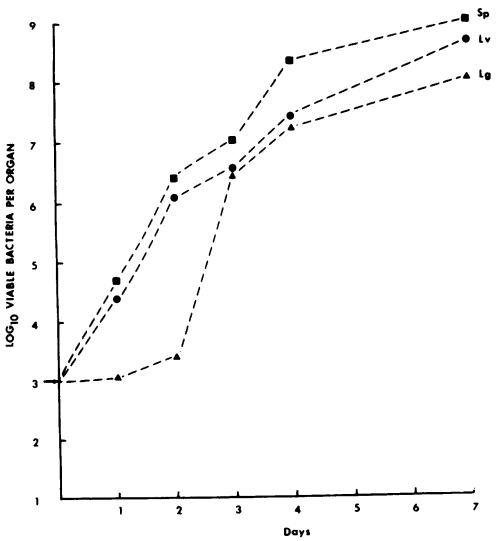

FIG. 2. *Growth of Y. enterocolitica WA after intravenous challenge of CD-1 mice. Abbreviations: Sp, spleen; Lv, liver; Lg, lung.*

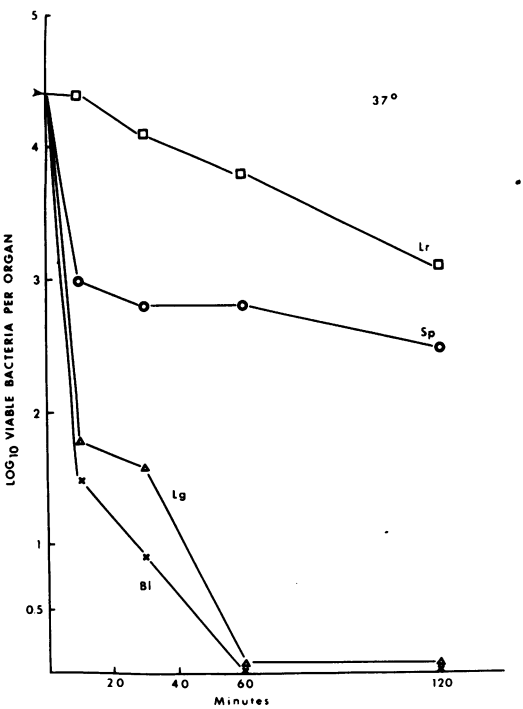

FIG. 3. *Growth curve in CD-1 mice for intravenous inoculum of Y. enterocolitica WA that been grown at 37 C prior to use. Abbreviations: Lr, liver; Sp, spleen; Lg, lung; Bl, blood. Arrowhead represents challenge dose.*

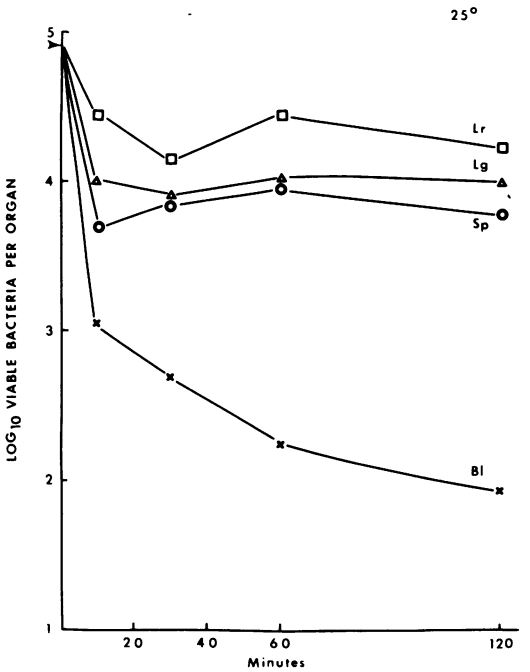

FIG. 4. *Growth curve for 25 C grown Y. enterocolitica WA in CD-1 mice after intravenous challenge.*

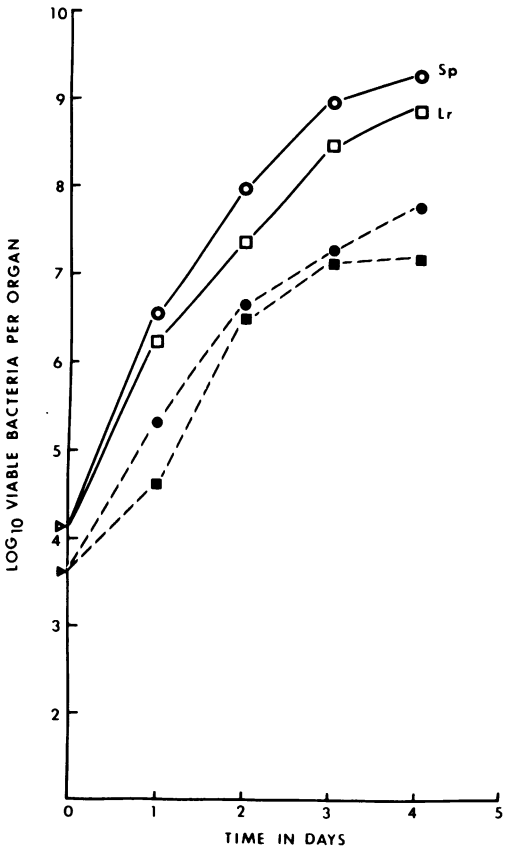

FIG. 5. *Growth of Y. enterocolitica WA cultured at 25 C (open symbols) and 37 C (solid symbols) in CD-1 mice after intravenous challenge.*

temic infection had become well established. Thus, except for the initial increase in the rate of phagocytosis and early killing of the 37 C grown organisms, the only difference between the two infections was the consistently higher bacterial count in those animals infected with 25 C grown bacteria. *Y. enterocolitica* WA cultured at 5 C gave rise to an in vivo growth curve identical to that seen with the 25 C grown cells and was therefore not included in Fig. 4 and 5. The small difference in the size of the respective challenge inocula was not responsible for the difference in the growth curves because the same phenomenon was observed when mice were challenged with the same size inocula or with higher numbers of 37 C grown organisms.

This temperature-related phenomenon was again observed when intraperitoneal clearance and killing was investigated. Although 90% of the opsonized bacteria in both groups were taken up by peritoneal cells over a very short time, negligible killing occurred when bacteria cultured at 25 C were injected, whereas the bacterial counts with the 37 C grown cells decreased 10-fold over a 60-min period (Fig. 6).

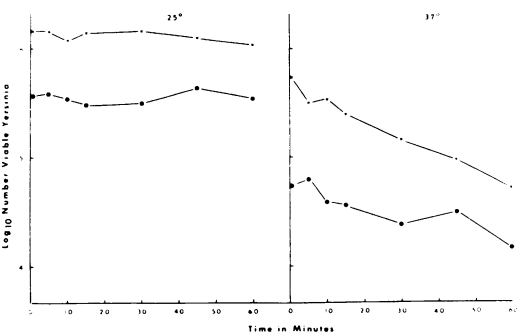

FIG. 6. *Peritoneal clearance and killing of Y. enterocolitica WA grown at 25 and 37 C. Symbols: ×, total number of viable Y. enterocolitica WA; ●, viable Y. enterocolitica WA that are not cell associated.*

DISCUSSION

The mouse is susceptible to infection with *Y. enterocolitica* strain WA whether the bacteria are administered orally, aerogenically, or intravenously. Appropriate challenge doses gave rise to progressive systemic infections, often resulting in death. The *Yersinia* growth curves in mice were very similar to those obtained earlier for another enteric pathogen, *Salmonella enteritidis* (6). Curiously, the susceptibility of

CD-1 and $C_{57}B1$ mice to *Y. enterocolitica* infection was the reverse of that seen with *S. enteritidis*, where the $C_{57}B1$ strain, which is exquisitely susceptible to salmonellosis (8), is more resistant to *Yersinia* infection than the CD-1 strain. The very high bacterial load that the *Yersinia*-infected animal can carry in its tissues is another distinguishing feature of this disease compared with murine typhoid. Mice infected with *S. enteritidis* frequently die when the number of viable salmonellae that can be recovered from their livers and spleen reaches about 10^8 viable organisms (7). Mice infected with *Y. enterocolitica*, on the other hand, may not succumb even when the number of viable yersiniae in the liver and spleen reaches nearly 10^{10}. This may merely be a reflection of the relative endotoxin contents of the two organisms, assuming that death of both *Yersinia*- and *Salmonella*-infected animals can be attributed to endotoxicosis (P. B. Carter, submitted for publication). If this is so, then the endotoxin level in the heavily infected animal may not reach lethal proportions until the number of yersiniae in the tissues surpasses 10^9; only one-tenth as many salmonellae may yield such a lethal level of endotoxin in mouse typhoid.

The tremendous variation seen in the extent and severity of the *Yersinia* infections in minimally infected mice was largely overcome simply by increasing the size of the challenge dose. Even so, variation in the number of viable yersiniae can still occur between different organs in the same animal, and this may be related to the tendency of the organism to give rise to well-organized abscesses. Thus, very high colony counts may occur in one organ with only a single large abscess, whereas another organ, or even another selected portion of the same organ, in the same animal may contain few or no bacilli. The lower counts exhibited by animals surviving past day 8 of the infection can be attributed to the elimination of innately susceptible mice from the host population rather than to an immune response. In fact, the existence of an immune response, effective in combating and effectively protecting against *Y. enterocolitica* infection, remains to be demonstrated.

The reason for the virulence of the WA strain for normal mice, in the face of the complete lack of pathogenicity by other strains (17), is still obscure. *Y. enterocolitica* strain 5819 is identical to the WA strain in terms of colonial morphology, biotype, and serotype (4). However, *Y. enterocolitica* 5819 completely lacks the ability to set up a persisting infection in the murine host; an intravenous challenge dose of 10^6 organisms is destroyed very quickly, and no viable bacteria are detectable in the tissues at 48 h. The possibility that a lysogenic phage was responsible for the introduction of unknown virulence factors in the WA strain was investigated, but no evidence for such a phage could be demonstrated (4). Likewise, the existence of a novel exotoxin or a particularly potent endotoxin in the WA strain could not be demonstrated (P. B. Carter, submitted for publication).

Y. enterocolitica WA is capable of in vitro growth over a broad temperature range (4), but at some point above 27 C, the organism becomes nonmotile, a phenomenon that appears to be characteristic of the *Y. enterocolitica* group as a whole (16, 18). The higher cultivation temperatures also result in decreased virulence that correlates with the enhanced inactivation of the challenge inoculum by the normal host. At selected challenge doses, the difference in the virulence of the 25 and 37 C cultures is also expressed in terms of morbidity and mortality. The septicemia resulting from an intravenous inoculum of organisms cultured at 25 C was longer lived than that caused by the 37 C grown organisms, suggesting that the enhanced virulence of the yersiniae cultured at lower temperatures was related to its resistance to phagocytosis. However, peritoneal clearance data indicated that an equal percentage of both the 25 and 37 C grown organisms became cell associated over the 5-min period of phagocytosis, but the 25 C cultured organism then appeared to be more resistant to the intracellular killing. The precise characteristic that is responsible for the increased resistance to in vivo inactivation of the 25 C grown *Y. enterocolitica* is not known. This finding is very similar to that reported for *Y. pestis* (3, 5, 10, 11), and the mechanisms could be the same. A relationship of the in vitro culture temperature to host virulence has been described for other species of *Yersinia*, but it is interesting to note that the relationship described has been just the inverse of that found for *Y. enterocolitica* (2). Such an inversion, allowing for greater virulence at lower temperatures, may account for the predominance of *Y. enterocolitica* infections in colder climates.

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